Review Report submitted to U.S. Fish and Wildlife Service 27 January 2006

"Comprehensive analysis of molecular phylgeographic structure among the meadow jumping mice (*Zapus hudsonius*) reveals evolutionary distinct subspecies."

By King et al.

Overview

Molecular genetic diversity among five subspecies of *Z. hudsonius* was examined by fragment analysis of 21 nuclear microsatellite DNA loci and sequence analysis of 1380 base pairs of mitochondrial (mt) DNA sequence. A total of 320 specimens were collected from 13 locations, with an average of 24 specimens per locality (range 14-34). One subspecies (*Z. h. preblei*) was sampled from 6 locations, three (*Z. h. intermedius*, *Z. h. campestris,* and Z*. h. pallidus*) from 2 locations each, and one (*Z. h. luteus*) from a single location. In addition, 7 specimens of *Z. princeps* were included for outgroup comparison. For most samples, ear punches or blood were collected non-invasively from live trapped animals; additional samples represented archived tissues (ear punches or frozen liver). Routine protocols were used for DNA extraction, PCR amplifications, electrophoresis and scoring of raw data. Recommended precautions were followed, such as negative controls in PCR reactions (to check for contamination), forward and reverse sequencing of DNA strands (to verify base calling), and independent scoring by two different individuals (to detect scoring errors). Standard analytical procedures were applied to the different molecular datasets and Bonferroni adjustment applied where appropriate. Microsatellite DNA data were first tested for deviation from Hardy-Weinberg expectations and linkage disequilibrium before calculating standard genetic diversity indices (e.g., allelic richness). Assignment test was used first to allocate individuals probabilistically to pre-defined groups (i.e., subspecies), then to non-delineated (i.e., genetically distinct) clusters. Genetic distances (*D*a) were calculated between each pair of collections and subspecies, and clustered with neighbor-joining and multidimensional scaling. AMOVA was used to test hierarchical structure of genetic variation. MtDNA data were tested for rate heterogeneity and for best model of sequence evolution prior to analyses. Molecular diversity indices (haplotype and nucleotide diversity) were calculated. Genealogical relationships were assessed with maximum parsimony and maximum likelihood analyses. Again, AMOVA was used to examine hierarchical structure of genetic variation, by either considering haplotype frequencies only (as in traditional F_{ST}) or by taking molecular information into account (i.e., Φ_{ST}). Results describe distinct molecular clades congruent with subspecies designations, and reveal additional population substructure in at least two subspecies.

General Comments:

As the title suggests, this is a comprehensive assessment of molecular diversity within and among subspecies of *Z. hudsonius*. It is a well-done, thoroughly planned and meticulously executed study. Molecular methods and analytical protocols are clearly explained and descriptions contain sufficient detail. Sampling is thorough, molecular approaches technically competent, and appropriate analytical procedures were applied. Genetic patterns were examined by a variety of approaches, and tested for congruence among data sets and methods. Findings are assiduously discussed and interpretations stem from results and do not extend beyond the data. Comparisons to a previous study are factual, impartial and straight-forward. Authors do a good job at illuminating potential reasons for discrepancies between theirs and the Ramey et al. (2005) findings.

Minor comments:

Lines 574-578 This is semantics: The null hypothesis is not "supported" by data, but instead can or cannot be rejected by findings.

Table 2 Header should probably read: "Collection information on specimens from five neighboring subspecies…" (not four)

(1) Were appropriate methodologies and markers used? - Yes.

Sampling: A concerted effort was made to collect tissues from live-trapped specimens. This allowed (a) unambiguously identified sampling locale for each specimen, (b) each collection (population) to be evaluated with a sufficient number of individuals so as to assess within-population diversity, (c) each subspecies to be represented by at least two collections so as to compare genetic diversity among groups, and (d) sufficient amounts of high-molecular weight DNA to be extracted. Also, sample sizes are sufficiently large to appropriately reflect diversity found at microsatellite loci; allelic diversity of loci is moderate (3.3 – 7.0; Table 2) and differences among collections are not due to stochastic sampling errors (i.e., if too few individuals are collected, allelic diversity would not be properly represented in each population and differences could be simply due to sampling errors).

Molecular markers: Both nuclear and mitochondrial DNA markers were examined. This had several advantages: (a) genetic diversity across the genome was examined, (b) markers differ in their evolutionary rate and thus provide insights into different aspects of an organism's evolutionary history, and (c) different analytical protocols can be employed. Further, a considerable number of microsatellite loci (n=21) was examined, as was a substantial amount of base pairs across two different mtDNA regions. Both are appropriate markers to examine genetic structure among closely related, recently diverged taxa, such as subspecies.

Analytical methods: Analyses are thorough and take advantage of recent advances (such as Assignment Test or Statistical Parsimony). Multiple approaches are used to examine genetic patterns, and results based on different methods are compared. Overall, results are consistent among different approaches, and where discrepancies occur, authors provide plausible explanations as to why this might be.

(2) Are authors' conclusion about taxonomic validity of *Z. h. preblei* and neighboring subspecies supported by the data presented in the report? - Yes.

The authors provide multiple lines of evidence in the form of different molecular markers and various analytical approaches to support their conclusions. Results and interpretations are indeed convincing. The data clearly show genetic differentiation among the five subspecies, and further reveal genetic substructure within two of these. Assignment probabilities for allocation to subspecies are high, and even higher if population substructure is taken into account (seven clusters; Table 3). AMOVA reveals considerable genetic variation partitioned among subspecies, particularly so when molecular information is taken into account beyond simple haplotype or allele frequencies (Table 9). This supports the presence of genetically distinct and identifiable groups. Phylogenetic analyses (MP and ML) reveal distinct clades, albeit

evolutionary relationships between Z*. h. preblei* and *Z. h. intermedius* are not completely resolved (Fig. 7). However, the latter reflects relative recent divergence of these two subspecies, and suggests the mtDNA regions examined provide insufficient resolution. Shallow divergence is also suggested by large haplotype and low nucleotide diversity within populations (Table 7). Further, no haplotypes are shared among subspecies, and haplotype networks clearly reveal independent evolutionary trajectories among subspecies (Figs. 5 and 6).

(3) Are author's conclusions that *Z. h. preblei* is comprised of at least two distinct population segments worthy of individual management supported by the data presented in the report? - Yes.

Multiple lines of evidence support this conclusion: (a) higher assignment probabilities if seven rather than five clusters are considered (Table 3; seven clusters reflecting population substructure within subspecies *Z. h. preblei* and *Z. h. intermedius*); (b) larger genetic distances between collections from northern and southern clusters as compared to genetic distances within each (Table 4); (c) AMOVA reveals larger percentage of variance distributed among-populations if only five clusters are considered (i.e., subspecies) as compared to seven; and (d) lack of shared haplotypes for CytB between northern and southern populations of *Z. h. preblei* (Appendix C).

(4) Possible alternative interpretations: could such be drawn from the genetics data? If so, how likely are these possibilities?

Authors discuss alternative conclusions and provide explanations why some are more plausible than others. Thus, they evaluated those alternative interpretations that could be reasonably assumed, and supported their conclusions with fact-based arguments.

One alternative interpretation could be: Population substructure identified in *Z. h. preblei* could reflect clinal variation and might appear distinct due to insufficient sampling (i.e., only extremes of the cline were sampled and not intermediates). However, this is highly unlikely since sampling locations appear relatively equidistant and continuous along Front Range, as far as the patchy distribution of *Z. h. preblei* permitted.

(5) Additional analyses: are any needed to verify the study's assertions and why?

A comprehensive array of analyses was already employed, and data were analyzed in a variety of ways. A few minor aspects that could be considered (but are unlikely to change any conclusions) are: (a) test mtDNA genes for selection to verify that indeed independent evolutionary histories and not selective pressures led to genetic differences among subspecies/clusters; (b) evaluate microsatellite data with program MicroChecker to test for null-alleles (i.e., mutation in primer annealing site that causes amplification failure of particular alleles, thus leading to homozygote excess). However, data were tested for, and did not deviate from, Hardy Weinberg expectations, making null-alleles unlikely. Also, since most loci were developed for the study species (*Z. hudsonius*) with reminder from closely related species, null-alleles are unlikely.

Authors suggest that close relationships between *Z. h. campestris* and *Z. h. intermedius* should be examined in more detail, and thus warrant further study. However, this would not alter conclusions about distinctiveness of *Z. h. preblei*.

(6a) Conflicting conclusions of Ramey et al. (2005) and King et al. (2006): What are most likely explanations?

King et al. (2006) provide a detailed discussion as to why their findings/conclusions differ from the ones made by Ramey et al. (2005). Most reiterate concerns I raised in my review of the reports provided to USFWS by Ramey et al. These include: (a) insufficient sampling and lack of proper representation of within versus among population diversity; (b) reliance upon museum specimens and problems associate with "Ancient DNA" work (e.g., low DNA yields that increase potential of contamination, while degraded DNA only permits amplification of short fragments and makes more probable the erroneous incorporation of nucleotides during early PCR cycles); (c) analysis of an insufficient fragment of mtDNA Control region that was clearly insufficient to encapsulate shallow divergence between subspecies.

All of the above are addressed by King et al. (2006): (a) sampling was more comprehensive, including more individuals per collection and multiple collections per subspecies; (b) tissues were collected from live-trapped animals, resulting in higher yields and quality of DNA, making amplification more robust and reliable; (c) analyses of substantially larger numbers of microsatellite loci, more base pairs of sequence data, as well as examination of two instead of one mtDNA region.

More samples and more data allowed King et al. (2006) to employ are variety of analytical methods for the examination of evolutionary history and population structure of the study taxa. In turn, multiple lines of evidence were provided to support particular interpretations of findings, and underscore high probability of conclusions.

(6b) Does new information change conclusions regarding synonymizing of *Z. h. preblei* and neighboring subspecies?

Ramey et al. (2005) provided insufficient data to draw conclusions about distinctiveness of *Z. h. preblei*. Thus, their findings neither supported nor rejected the notion of genetic distinctiveness of *Z. h. preblei* as a unique subspecies, and their suggestion to synonymize *Z. h. preblei* with neighboring subspecies went far beyond their data. In contrast, data provided by King et al. (2006) document genetic differences, albeit shallow, between *Z. h. preblei* and other subspecies. The analyses of King et al. reveal genetic distinct clusters that are congruent with previous subspecies designations based on morphological data. In light of these new findings, the synonymization of *Z. h. preblei* with other subspecies is not warranted and cannot be recommended.